Effect of Roux-en-Y gastric bypass and diet-induced weight loss on diabetic kidney disease in the Zucker diabetic fatty rat

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Abstract: BACKGROUND Reductions in urinary protein excretion after Roux-en-Y gastric bypass (RYGB) surgery in patients with diabetic kidney disease have been reported in multiple studies. OBJECTIVES To determine the weight loss dependence of the effect of RYGB on urinary protein excretion by comparing renal outcomes in Zucker diabetic fatty rats undergoing either gastric bypass surgery or a sham operation with or without weight matching. SETTING University laboratories. METHODS Zucker diabetic fatty rats underwent surgery at 18 weeks of age. A subgroup of sham operated rats were weight matched to RYGB operated rats by restricting food intake. Urinary protein excretion was assessed at baseline and at postoperative weeks 4 and 12. Renal histology and macrophage-associated inflammation were assessed at postoperative week 12. RESULTS Progressive urinary protein excretion was attenuated by both RYGB and diet-induced weight loss, albeit to a lesser extent by the latter. Both weight loss interventions produced equivalent reductions in glomerulomegaly, glomerulosclerosis, and evidence of renal macrophage infiltration. CONCLUSION Weight loss per se improves renal structure and attenuates renal inflammatory responses in an experimental animal model of diabetic kidney disease. Better glycemic control post-RYGB may in part explain the greater reductions in urinary protein excretion after gastric bypass surgery.

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Effect of Roux-en-Y Gastric Bypass and Diet induced Weight Loss on Diabetic Kidney Disease in the Zucker Diabetic Fatty Rat.

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Running Title: Intensive Weight Loss and Diabetic Kidney Disease

Keywords: RYGB, gastric bypass, bariatric, diabetic kidney disease, weight loss, renal inflammation, proteinuria, Zucker Diabetic Fatty rat.

Contribution statement

KJN, JAE, SNJ, CC, and KA completed the animal experiments and data analysis. KJN wrote the first draft of the paper. CB redrafted the paper. CG, TAL, NGD, CWlR designed the experiments and redrafted the paper.

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Abstract

Background:

Reductions in urinary protein excretion following Roux-en-Y gastric bypass (RYGB) surgery in patients with diabetic kidney disease (DKD) have been reported in multiple studies.

Objectives:

To determine the weight loss dependence of the effect of RYGB on urinary protein excretion by comparing renal outcomes in Zucker Diabetic Fatty rats (ZDF) undergoing either gastric bypass surgery or sham operation with or without body weight matching.

Setting:

University laboratories.

Methods:

ZDF rats underwent surgery at 18 weeks of age. A sub-group of Sham operated rats were body weight matched to RYGB operated rats by restricting food intake. Urinary protein excretion was assessed at baseline and at post-operative weeks 4 and 12. Renal histology and macrophage-associated inflammation were assessed at post-operative week 12.

Results:

Progressive urinary protein excretion was attenuated by both RYGB, and diet-induced weight loss albeit to a lesser extent by the latter. Both weight loss interventions produced equivalent reductions in glomerulomegaly, glomerulosclerosis and evidence of renal macrophage infiltration

Conclusion:

Weight loss per se improves renal structure and attenuates renal inflammatory responses in an experimental animal model of DKD. Better glycemic control post-RYGB may in part explain the greater reductions in urinary protein excretion following gastric bypass surgery.

Keywords: RYGB, gastric bypass, bariatric, diabetic kidney disease, weight loss, renal inflammation, urinary protein excretion, Zucker Diabetic Fatty rat.

Introduction

In the classical model of diabetic kidney disease (DKD) progression, incipient nephropathy characterised by progressive microalbuminuria and initial hyperfiltration gives way over a 10-20 year period to overt urinary protein excretion and accelerated decline of renal function as assessed by glomerular filtration rate [1]. The progression of disease is associated with renal inflammation and is driven by hypertension and hyperglycaemia. Despite increased use of multimodal therapeutic regimens targeted at pharmacological remediation of these vascular risk factors, DKD still often follows an inexorable course towards end stage renal disease requiring dialysis or transplantation [2].

Roux-en-Y gastric bypass (RYGB) is now increasingly considered as an option for treatment of type 2 diabetes, in combination with medical therapy, in those patients not meeting treatment targets or with diabetic complications [3, 4]. Surgery is more effective than medical therapy alone at inducing weight loss and improving glycaemic control [4]. RYGB also rapidly reduces urinary protein excretion in patients with type 2 diabetes [5, 6]. This is coincident with evidence of remediation of renal inflammation as measured by markers of pro-inflammatory activity such as urinary monocyte-chemoattractant protein-1 (MCP-1) [5 6].

Improvements in urinary protein excretion and renal inflammation correlate with the degree of post-operative weight [5 6]. Weight loss *per se* may therefore drive remediation of DKD. Although a direct causal role for reductions in adiposity seems plausible, it remains speculative in the absence of comparative studies examining the relative impact of matched surgical and non-surgical weight loss. The relative importance of the weight loss component of RYGB is challenging to test in clinical studies, as it is difficult to produce weight loss equivalent to RYGB using a non-surgical treatment in humans [7].

Using the Zucker Diabetic Fatty (ZDF) rat model of DKD [8] we recently demonstrated that a 12 week dietary restriction regimen tailored to deliver RYGB-equivalent weight loss reduced total body weight in ZDF rats by approximately 20% relative to *ad libitum* fed rats [9]. We used this model in the present study to compare the relative impact of both RYGB and equivalent weight loss achieved through caloric restriction on DKD in the ZDF rat (Figure 1).

Methods

Animals

ZDF rats are homozygous null mutant for the long isoform of the leptin receptor and develop hyperphagia, progressive obesity, diabetes and renal microvascular complications [8]. Ten-week-old male ZDF rats (fa/fa) (n=29) (Charles River Laboratories, France) and heterozygote non-obese, non-diabetic healthy control ten-week-old ZDF rats (fa/+) (n=5) were group housed in a temperature and humidity controlled room with a 12-hour light/dark cycle (lights on from 02.00 to 14.00). Rats had free access to tap water and Purina Lab diet #5008 (Purina Mills, St. Louis, MO) throughout the protocol, except where otherwise noted. All experimental procedures were approved by the Veterinary Office of the Canton Zurich, Switzerland and complied with national laws and current ethical guidelines.

The surgical procedures have been previously described [9]. Briefly, in the RYGB the jejunum was dissected 60 mm distal from the ligament that attaches the jejunum to the colon transversum. A 7-mm side-to side small bowel anastomosis was performed between the biliopancreatic limb and the lower jejunum 250–300-mm proximal to the cecum to create the common channel. After exposure and careful mobilization of the gastro-esophageal junction,

the stomach was transected just below the gastro-esophageal junction to create a small gastric pouch about 2% of the original stomach size. The stomach remnant was subsequently closed, and the small gastric pouch was anastomosed end-to-side to the alimentary limb, which was ~500 mm in length.

For the sham surgery, the entire gastrointestinal tract was mobilized and then a 10-mm gastrotomy was performed on the anterior wall of the stomach with subsequent closure in two layers. A 7-mm jejunotomy was then performed and subsequently closed.

Glycaemic Control Monitoring

Blood glucose was measured in all rats four times weekly with a point-of-care glucometer (Breeze2, Bayer, Zurich, Switzerland). Using these values, a Glucose Area Under the Curve (AUC) was calculated to evaluate glycaemic exposure over the course of the study. Urine was collected over 24 hours in a metabolic cage during the week before surgery (-1 week) and at 4 and 12 weeks post-operatively, respectively. Urine was stored at -20^{o}C.

Experimental Design (Figure 1)

ZDF (fa/fa) rats were randomly assigned to RYGB (n=15), Sham surgery and *ad libitum* diet (n=6), or Sham surgery and body weight matching (n=8). The RYGB group were not food restricted. The Body Weight Matched group was food restricted to match the weight loss of the RYGB group. Heterozygote controls (Non-obese Non-diabetic Controls, n=5) and the Ad libitum Sham group were maintained on *ad libitum* diet. The Non-obese Non-diabetic Controls did not receive any surgical or medical intervention.

All animal work was conducted in line with the *Guide for the care and use of laboratory animals*, Eighth edition (2011) under local ethics committee and national license approval. During the twelfth post-operative week renal tissue was obtained at harvest and processed for histology, immunohistochemistry and gene expression analyses.

Histology & Immunohistochemistry

Periodic Acid-Schiff (PAS) staining was performed on deparaffinised, rehydrated 4μM paraffin-embedded renal sections using a commercially available PAS staining system and protocol (395B-1KT, Sigma-Alrdich, Steinheim, Germany).

Glomerular morphometry

Using PAS stained slides, glomerular density was calculated as follows: glomerular density (μM^{-2}) = number of glomeruli captured/total cortical area analysed. Glomerular cross sections within these fields were then digitally traced at 40x and the mean glomerular cross-sectional area was calculated as follows: mean glomerular cross-sectional area = cumulative cross-sectional area/number of glomeruli captured.

Glomerulosclerosis

In order to compare the observed frequency of glomerulosclerosis between groups, PAS-stained renal sections were semi-quantitatively assessed by digital light microscopy at 40x. Briefly, partially (>25%) or completely sclerotic glomeruli within cortical fields were counted, and the number of sclerotic glomeruli expressed as a percentage of the total number of glomeruli assessed. Sclerosis was scored positively when mesangial expansion, increased

mesangial cellularity, adhesion formation, and capillary obliteration appeared present in at least one segment accounting for >25% of the total glomerular cross-sectional area [10, 11].

ED1 Immunohistochemistry

Immunohistochemical analysis was performed on 4μM paraffin-embedded renal sections. Slides were deparaffinised and rehydrated through xylene and alcohol. Endogenous peroxidases were quenched using 5% H_2O_2 in citrate buffer for 15 minutes. Heat-mediated antigen retrieval was performed in citrate buffer using a standard pressure pan for 15 minutes followed by rapid cooling.

The macrophage marker CD68 (ED1 antigen) was used to identify macrophages in renal sections. Slides were incubated for one hour at room temperature in 1/50 mouse monoclonal antibody to CD68 (ED-1, Ab31630, Abcam, Cambridge, United Kingdom), and rinsed thoroughly with 0.05% Tween-20 in phosphate buffered saline (PBS). Negative controls were performed by replacing the primary antibody with PBS. Secondary and tertiary antibodies (polyclonal rabbit anti-mouse immunoglobulin/HRP [P0260] and polyclonal swine anti-rabbit immunoglobulin/HRP [P0217] 1/100 in 10% normal rat serum/PBS [090710, all Dako, Glostrup, Denmark]) were then applied.

Slides were then rinsed in PBS and visualised using diaminobenzidine (K3468, Dako, Glostrup, Denmark) as a chromogenic substrate with a haematoxylin counterstain. A colour deconvolution algorithm targeting DAB was used to quantify the area stained positive for ED-1 within the captured cortical fields. This was expressed as a percentage of the total analysis area, and the percentage area ED-1 positive was then compared between study groups. Stained slides were digitally imaged using Aperio ScanScope (Leica Biosystems Imaging, Nussloch, Germany) and analysed using Aperio ImageScope (Leica Biosystems

Imaging, Nussloch, Germany). All histomorphometric analyses were performed by a single blinded investigator. For PAS and ED-1 stained slides consecutive rectangular cortical fields containing at least 100 glomeruli in total were selected for analysis from each animal.

Gene Expression Analyses

RNA was isolated from snap frozen renal cortical tissue using Trizol Reagent (Qiagen, Valencia, CA, USA). Following DNAse treatment and reverse transcription reactions (Superscript® II, Life Technologies), TaqMan quantitative real-time polymerase chain reactions were performed according to the manufacturer's instructions, using primer sets for CCL2 (MCP-1): Rn00566655_ml; available at: https://www.lifetechnologies.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-assays/taqman-gene-expression.html; Life Technologies, Carlsbad, CA, USA). 18S was used as an internal control and group data expressed as relative quantities using mean fa/+ group values as an internal calibrator via the ΔΔCT method.

Urinary Biochemistry

Urinary creatinine and total protein were measured in samples of 24 hour urine collections on an auto-analyser. A protein:creatinine ratio was then calculated as a normalised measure of urinary protein excretion. Urinary MCP-1 was determined using the Rat MCP-1 Instant ELISA kit (eBioscience; Vienna, Austria).

Statistics

All descriptive and inferential statistics were conducted using Prism (Graphpad Software, California, U.S.A.). Data are presented as mean ± SEM. One-way ANOVA with *post hoc* Bonferroni test was used to compare group data. Significance was set at $p<0.05$.

Results

A total of 8 rats in the RYGB group died post-operatively, 4 in the immediate post-operative period, and the remainder from 6 weeks post-operatively when they died of severe hypoglycaemia. There was no mortality in the other groups.

At baseline, all fa/fa rats (Ad libitum Sham, RYGB and Body Weight Matched) had comparable protein:creatinine ratios, which were elevated 5-6 fold relative to the Non-obese Non-diabetic Control group ($p<0.05$; Figure 2).

As previously described, RYGB and Body Weight Matched rats achieved equivalent weight loss, but plasma glucose levels did not improve among Body Weight Matched animals to the same extent as RYGB rats. The Body Weight Matched group had greater plasma glucose concentrations, with a cumulative post-operative glucose AUC of 1053mmol/L/day more than the RYGB group ($p<0.001$) [9].

There was a significant reduction in urinary protein excretion from baseline to week 4 post-RYGB ($p=0.01$) which was maintained to post-operative week 12. In the Body Weight Matched group, there was an initial reduction in urinary protein excretion at post-operative week 4 ($p=0.02$), although the absolute protein:creatinine ratio was on average 2-fold higher than that observed in the RYGB group ($p=0.009$) at the same time-point. By post-operative week 12, urinary protein excretion in the Body Weight Matched group increased but

remained reduced relative to the *Ad libitum* Sham group (p=0.04). No change in urinary protein excretion from baseline to post-operative week 4 was seen in *Ad libitum* Sham rats (age 21 weeks), but a significant 3-fold increase followed between post-operative weeks 4 and 12 (age 30 weeks) (p<0.01).

Sclerotic lesions were reduced by 30-50% post--RYGB (p=0.01) and Body Weight Matched (p=0.03; Figure 3A, 3B). The impact of RYGB and Body Weight Matched was similar (p=0.34). Glomerular tuft size was increased on average by 39% in the *Ad libitum* Sham versus the Non-obese Non-diabetic Control group (p<0.001, Figure 3C). No difference in tuft size was observed between the RYGB and Body Weight Matched groups (p=0.17). The *Ad libitum* Sham group showed evidence of DKD including focal sclerosis (arrow in Figure 3A panel ii) and patchy tubulointerstitial inflammation. In 30% (+/- 3%) of glomeruli (Figure 2B) focal sclerosis occurred alongside tubular dilatation in the *Ad libitum* Sham group.

Renal macrophage infiltration (Figure 4A and B), urinary MCP-1:creatinine ratio (Figure 4C) and renal MCP-1 mRNA expression (Figure 4D) were all significantly attenuated by RYGB and Body Weight Matching. There was no significant difference in effect between RYGB and Body Weight Matching. Conversely, renal macrophage infiltration, urinary MCP-1:creatinine ratios and MCP-1 expression were all markedly elevated in the *Ad libitum* Sham group relative to the Non-obese Non-diabetic Control, RYGB and Body Weight Matching groups (all p<0.01.)

Discussion

The present study set out to assess the relative impact of equivalent surgical (RYGB) and non-surgical (dietary restriction in the Body Weight Matched group) weight loss on the

progression of DKD in the ZDF rat. The clinical relevance of the study stems from the increasing use of surgical approaches such as RYGB as a means of correcting the diabetic milieu and potentially arresting the onset or progression of complications of diabetes such as DKD [4, 7, 12].

There has been some concern regarding the use of bariatric surgery in kidney disease given the potential for complications such as nephrolithiasis [13]. However, the weight loss effect may be of significant benefit. Unhealthy visceral fat stores have been directly implicated in renal injury secondary to reduced production of renoprotective adipocytokines such as adiponectin and simultaneous increases in the release of pro-inflammatory systemic signals [14]. Therefore a reduction in fat mass could concievably remediate the pro-inflammatory activity contributing towards renal injury in DKD. Establishing whether weight loss *per se,* when comparable in magnitude to that obtained following bariatric surgery, has a direct impact on DKD outcomes constitutes an important question for translational bariatric research in kidney disease.

Diet-induced weight loss equivalent in magnitude to that obtained following RYGB is less efficacious than surgery in reducing hyperglycaemia in the ZDF rat [9]. Given the prevailing idea that hyperglycaemia drives both renal inflammation and injury, we hypothesised that equivalent weight loss without correction of hyperglycaemia would be less efficacious than RYGB as a means of arresting DKD progression.

Interestingly, significant weight loss achieved through dietary restriction in our Body Weight Matched group had an equivalent impact on glomerular morphology and markers of renal macrophage inflammation as compared to RYGB despite the persistence of hyperglycaemia. Thus the present findings suggest that adiposity *per se* is a major driver of renal inflammation and glomerulomegaly in DKD, and that weight loss is an important mediator of

improvements in DKD post-RYGB. This would suggest that weight loss alone could remediate renal inflammation in DKD.

Nonetheless, in the present study RYGB did produce superior improvements in urinary protein excretion as compared with equivalent non-surgical weight loss. Given that there was greater hyperglycemia in the Body Weight Matched group, one explanation may be that the hyperglycemia itself promoted dysfunction at an ultrastructural level facilitating excess urinary protein excretion. Electron microscopy based analysis of the glomerular filtration barrier would have permitted further investigation of potential ultrastructural explanations for these divergent results.

Other inflammatory mediators may also be important at the filtration barrier. The effect of inflammation at the level of the podocyte is incompletely understood. There is a negative correlation between plasma adiponectin and kidney dysfunction in obese populations[15-17]. Adiponectin knockout mice have abnormal podocyte morphology[15]. This altered morphology is associated with functional deficits at the filtration barrier, and increased urinary protein excretion.

In a high fat fed rat model with strepozotozin induced diabetes, an extended RYGB can enhance the expression of anti-inflammatory factors [18]. This change is seen in parallel with improvements in insulin sensitivity and reduced urinary protein excretion. RYGB specific factors affecting metabolic control such as enhanced incretin responses may also contribute to imrpovements in kidney disease [19]. Some of the renoprotective effects of incretin hormones such as glucagon-like peptide 1 have been shown to proceed independently of hyperglycaemia in pre-clinical models [20].

Therefore, there are multiple agents which are all likely to work in concert to produce improvements in DKD following weight loss or RYGB. Our results suggest that weight loss alone is sufficient to remediate renal inflammation, and can attenuate the progression of urinary protein excretion. However, RYGB has a greater and more durable effect on reducing urinary protein excretion in this study. The combination of enhanced glucagon-like-1 peptide excretion, reduced hyperglycemia and improvements in the inflammatory millieu may explain the greater effect of RYGB on urinary protein excretion. However, this remains speculative at this point, and further translational work in human randomised clinical trials is now needed to determine the role of bariatric surgery in the management of DKD.

Conclusion

Diet-induced weight loss equivalent to that achieved by RYGB can produce equivalent reduction in renal inflammation in diabetic kidney disease. However, RYGB has a greater effect on reducing urinary protein excretion. The greater effect of RYGB on glycaemic control as compared to diet-induced weight loss may explain this discrepancy. To replicate the beneficial effects of RYGB on DKD with non-surgical methods may thus require combining intensified weight loss with pharmacological correction of hyperglycemia.

Conflict of interests

None of the authors have any conflict of interests to declare.

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Figure Legends

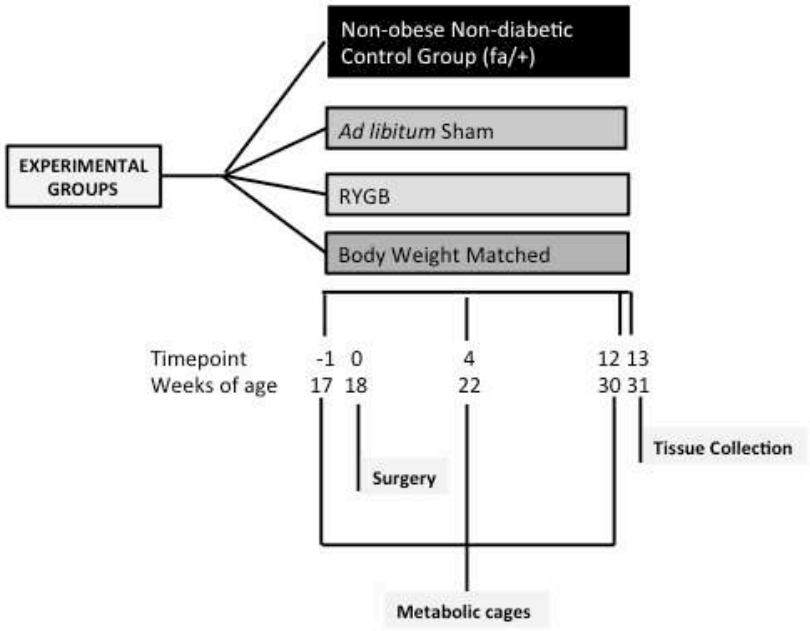

Figure 1: Experimental Design

Figure 1-Experimental design

Homozygous Zucker Diabetic Fatty rats (ZDF-fa/fa) were allocated to RYGB (n=15) or sham surgery comprising intestinal transection and re-anastomosis (n=14). In the sham surgery group, rats were either allocated to dietary restriction to induce weight loss equivalent to the RYGB group (Body Weight Matched, n=8) or allowed ad libitum access to food (Ad libitum Sham, n=6). Heterozygous Fa/+ animals served as non-obese and non-diabetic controls (n=5). Serial urine samples were collected 4 and 12 weeks after surgery prior to humane euthanasia at the start of the 13th post-operative week.

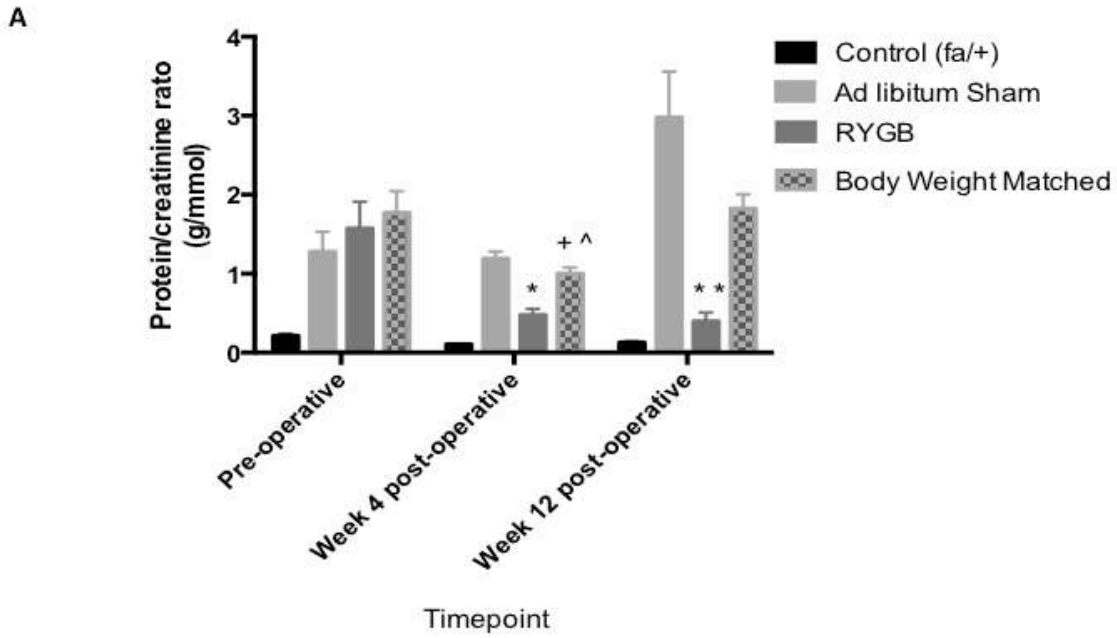

Figure 2:
Time dependent impact of RYGB or equivalent diet-induced weight loss (Body Weight Matching) on proteinuria in Zucker Diabetic Fatty rats
*p<0.05: RYGB Group 4 weeks Vs. Baseline **RYGB Group 12 weeks Vs. Baseline
+ p<0.05: Body Weight Matched Group 4 weeks Vs. Baseline
^p<0.05 RYGB versus BWM at 4 weeks

Figure 2 Time dependent impact of RYGB or equivalent diet-induced weight loss (Body Weight Matching) on urinary protein excretion in Zucker Diabetic Fatty rats

Protein:creatinine ratios were measured in urine collections obtained at 17 weeks of age (Wk -1) 4 weeks post-operative (22 weeks of age) and one week before study close (Wk 12 weeks post-operative, 30 weeks of age)

*p<0.05: RYGB Group 4 weeks Vs. Baseline **RYGB Group 12 weeks Vs. Baseline

+ p<0.05: Body Weight Matched Group 4 weeks Vs. Baseline

^p<0.05 RYGB versus BWM at 4 weeks

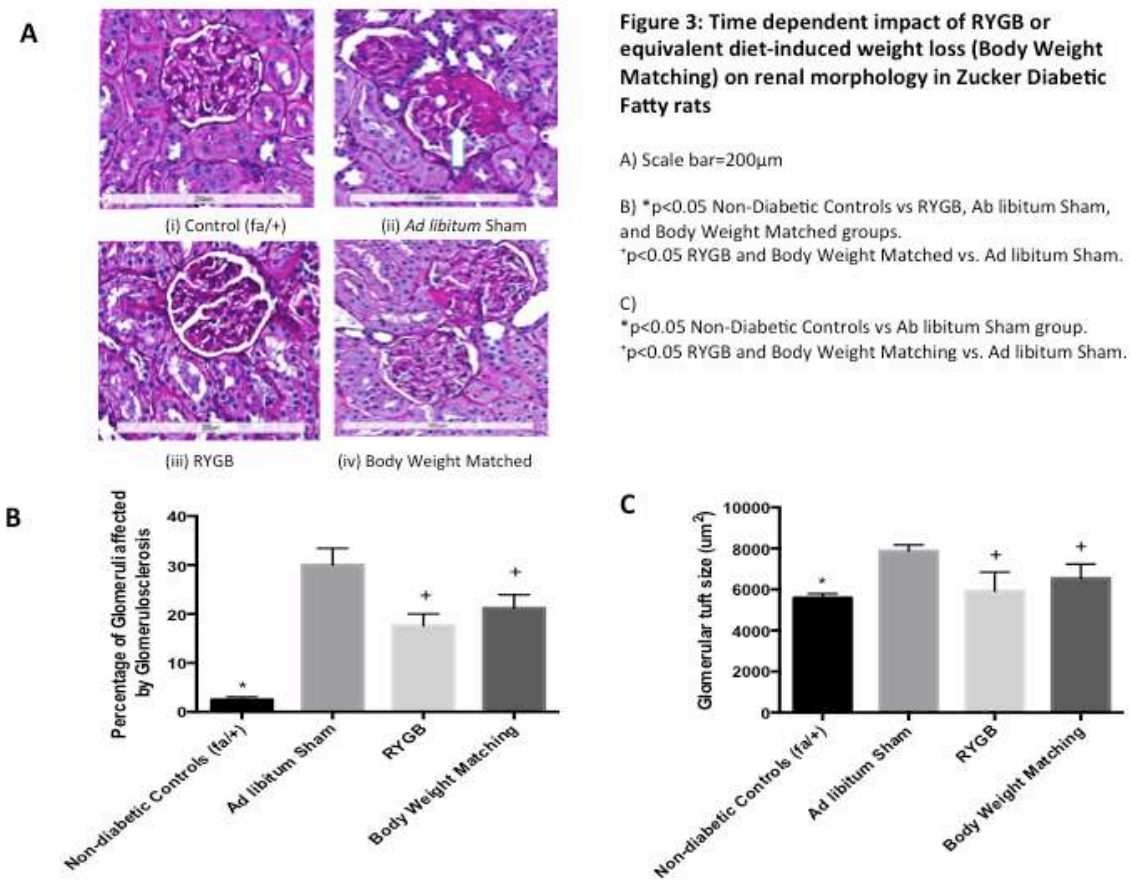

Figure 3 Time dependent impact of RYGB or equivalent diet-induced weight loss (BWM) on urinary protein excretion and glomerular morphology in Zucker Diabetic Fatty rats (*fa/fa*)

A) Four micron thick coronal sections of kidneys from Zucker Diabetic Fatty rats and heterozygote controls were stained with Periodic acid-Schiff and examined by medium power light microscopy. Samples from each group are shown.
(i) Non-obese Non-diabetic Control (*fa/+*) animal showing normal PAS positivity,
(ii) focal glomerulosclerosis in the *Ad libitum* Sham group (arrow)
(iii) relatively structurally preserved glomeruli post-RYGB
(iv) relatively structurally preserved glomeruli after diet-induced weight loss
Scale bar=200µm

B) Percentage of sclerotic glomeruli per high powered field (HPF) and mean glomerular tuft size

*p<0.05 Non-Diabetic Controls vs RYGB, Ab libitum Sham, and Body Weight Matched groups.

$^{+}$p<0.05 RYGB vs. Ad libitum Sham.

C) Tuft size is greatest in the Ad libitum Sham group and both RYGB and Body Weight Matching move tuft sixe towards normal

*p<0.05 Non-Diabetic Controls vs Ab libitum Sham group.

$^{+}$p<0.05 RYGB and Body Weight Matching vs. Ad libitum Sham.

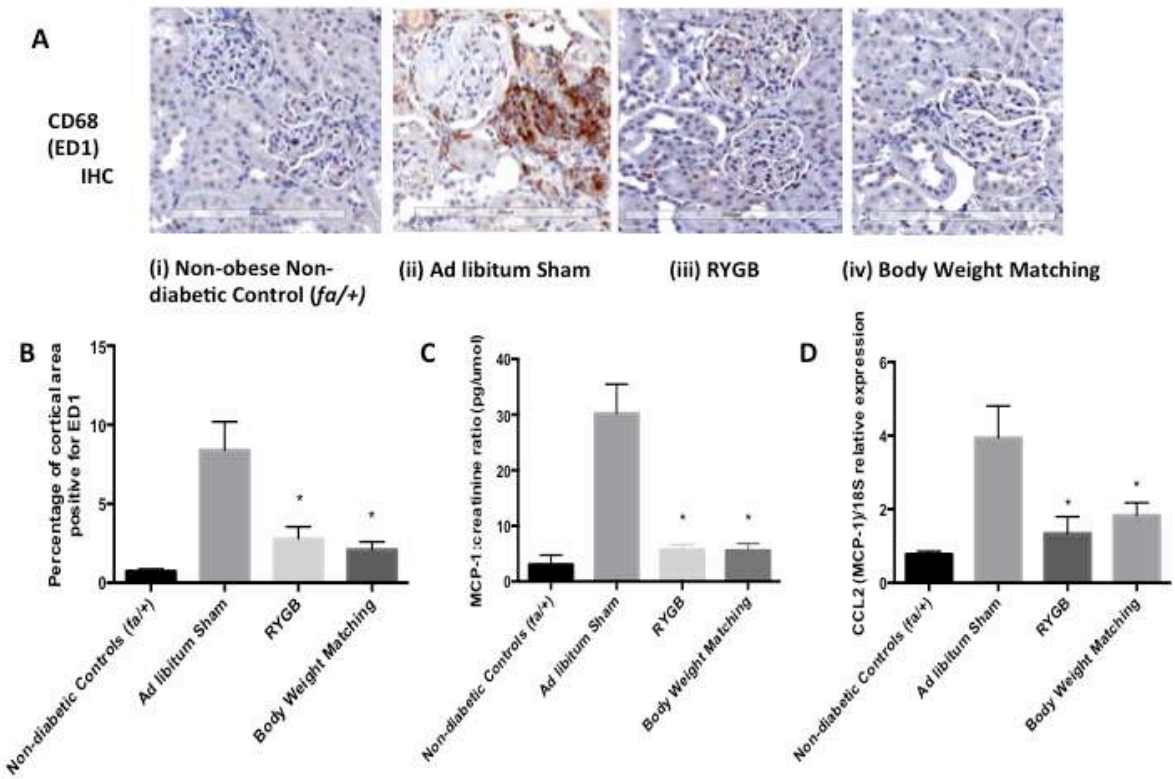

Figure 4: Effect of RYGB and equivalent diet-induced weight loss (Body Weight Matching) on renal macrophage Infiltration and urinary and tissue MCP-1 levels in ZDF rats

*p<0.05 RYGB and Body Weight Matchin vs. Ab libitum Sham

Scale bar = 200 µm

Figure 4 Effect of RYGB and equivalent diet-induced weight loss (BWM) on renal macrophage Infiltration and urinary and tissue MCP-1 levels in ZDF rats

A) Four micron thick coronal section of kidneys from Zucker Diabetic Fatty (ZDF) rats and heterozygote controls (*fa*/+) were stained for CD68 (ED1) to evaluate macrophage infiltration. Examples from each group are shown at medium power magnification;

(i) non-diabetic *fa*/+ animals with no significant ED-1 staining

(ii) extensive peri-glomerular macrophage infiltration (brown stained areas) in an ALS animal

(iii) reduced macrophage infiltration around the glomeruli post-RYGB

(iv) reduced macrophage infiltration around the glomeruli after diet-induced weight loss in the BWM group

Scale bar = 200 µm

B) The differences in ED1 area positivity as a percentage of cortical area was also calculated. *p<0.05 ALS versus all other groups).$^{+}$p<0.05 versus ALS C) Urinary MCP-1:creatinine ratios are compared at the final urinary collection of the study*p<0.05 ALS versus all other groups).$^{+}$p<0.05 versus ALS at 12 weeks post-operatively (age 31 weeks) D). Relative expression of MCP-1 mRNA was assessed in renal tissue *p<0.05 ALS versus all other groups).$^{+}$p<0.05 versus ALS